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<p>(21) International Application Number: PCT/EP89/00521 (22) International Filing Date: 12 May 1989 (12.05.89) (30) Priority data: 194,636 16 May 1988 (16.05.88) US (71) Applicants: SANDOZ AG [CH/CH]; Lichtstrasse 35, CH-4002 Basel (CH). VESTAR, INC. [US/US]; 650 Cliffside Drive, San Dimas, CA 91773 (US). (72) Inventors: KONIGSBERG, Paula, Jean ; 125 North Allen Avenue, Apt. 308, Pasadena, CA 91106 (US). RICHER, LeRoy, Leonard ; 6924 Willard Avenue North, San Gabriel, CA 91775 (US). SCHMIDT, Paul, Gardner ; 1730 Euclid Avenue, San Marino, CA 91108 (US). ULIANA, Joseph, Anthony ; 178 East Sierra Madre Boulevard, Sierra Madre, CA 91024 (US).</p>		<p>(74) Common Representative: SANDOZ AG; Patentabteilung, Lichtstrasse 35, CH-4002 Basel (CH). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i> <i>With amended claims.</i></p>
<p>(54) Title: LIPOSOMES COUPLED TO HORMONES (57) Abstract The invention relates to liposomes coupled to a hormone e.g. a cytokine in which the hormone retains specific binding activity to its cell membrane receptor and to a process for making them. The liposomes of the invention may be used as delivery vehicles for targeted delivery of a diagnostic or pharmaceutically active substance.</p>		

receptor on a given cell type may also be correlated with a malignant condition of the cells. Indeed some membrane receptors known to be normally present in small amounts are expressed at a much higher density on tumour cells, which may therefore be removed by specific targeting of cytotoxic drugs.

However, many of the above treatments have undesirable side effects due to the fact that the cytotoxic effect usually extends beyond the particular cell type involved in allograft rejection or in causing the disorder. For example, an essential problem arising from the use of some known immunosuppressive drugs is that the immunosuppressive action makes the patient particularly susceptible to infection by bacteria or viruses that would be controlled by a normal immune system. Another problem more especially associated with the delivery of cytotoxic drugs as conjugates with antibodies is that the antibody may not be internalized into the cells. Thus, although the toxin-antibody conjugate exhibits specific binding, it fails to deliver the toxin to the unwanted cells. As a result of the low frequency of internalization, impractically high concentrations of toxin-antibody conjugates must be used with corresponding increased risk of overall toxicity.

Thus, there is still a need for providing a therapeutic agent which can block or lyse unwanted cells at a high level of specificity and efficacy. It has been now found that these requirements may be achieved by encapsulating a cytotoxic drug into liposomes and linking the drug-containing liposomes to hormones. By "hormone" is meant a protein or a peptide which has a cell receptor binding site and which is able to induce a metabolic response in the target cell upon binding to its receptor. This includes, e.g. endocrine hormones which are conveyed in vivo by the blood from one organ to another as well as paracrine hormones and cytokines which are cell-to-cell mediators.

Accordingly, the present invention provides liposomes which are coupled to a hormone having a biologically active cell receptor binding site which is available for interaction with cells bearing

cytotoxic drugs for possible therapeutic use e.g. for preventing or treating allograft rejection episodes which are the result of a proliferative burst of activated T-cells induced by IL-2. It was anticipated that anti (IL-2 receptor) MAb conjugates could be effective in killing activated T-cells without adversely affecting the resting T-cells which are constantly required for normal immune surveillance, e.g. for fighting infections. However, these MAb's (usually called anti-TAC MAb's) recognize only the low affinity receptor and thus fail to be internalized.

However, where the IL-2 molecule or an active fragment thereof is used for targeting, binding will be predominantly to the high-affinity receptor. Therefore, the advantages of drug-containing liposomes coupled to IL-2 over anti-TAC MAb conjugates are: internalization of the drug and highly specific targeting of activated T-cells.

Liposomes are closed spherical shell-like structures comprising a bilayer membrane enclosing an aqueous volume. The primary constituents of the bilayer membrane are amphipathic molecules which may include phospholipids of natural or synthetic origin. For use in the present invention, the liposomes comprise phospholipids in which the hydrophilic group is phosphatidylethanolamine. Preferably, they also comprise phospholipids in which the hydrophilic group is phosphatidylcholine. The hydrophobic groups associated with phosphatidylethanolamine or phosphatidylcholine may be a variety of saturated and unsaturated fatty acid moieties. Phospholipids dispersed in aqueous solution spontaneously form bilayers with the hydrocarbon tails directed inward and the polar headgroups outward to interact with water.

Most preferably, the liposomes for use in the present invention are partially or totally composed of distearoyl phosphatidylethanolamine (DSPE) and/or distearoyl phosphatidylcholine (DSPC) in various ratios. It is also preferred that a steroid such as cholesterol be present in the liposome membrane. Suitable weight

methotrexate; the last being particularly preferred.

With respect to cyclosporin A, (Sandimmune[®]) intravenous administration of the pharmaceutical compound in treatment of organ transplantation or graft-versus-host disease is normally at a dosage of 3-5 mg/kg/day. Oral doses are higher by approximately a factor of three. When administered using the delivery vehicles of the present invention, lower dosages may be expected to achieve comparable effects in view of the targeted delivery of the drug to immune system cells responsible for the disease conditions.

Methotrexate has been successfully encapsulated in amounts of 20-200 µg/mg lipid the concentration of which was then adjusted to produce 0.3mM methotrexate in aqueous liposome dispersion for administration.

According to another aspect of the invention, there is provided a process for linking liposomes to a hormone, having a biologically active cell receptor binding site.

Such a process comprises:

- a) fixing a coupling agent to liposomes and then reacting with the hormone, or
- b) fixing a coupling agent to the hormone and reacting with liposomes or,
- c) fixing a first coupling agent to liposomes, fixing a second coupling agent to the hormone and then reacting the coupling moieties together,

the alternative process c) being particularly preferred.

It may also be desirable to introduce a linker arm having 4 to 6 carbon atoms between the liposome and the ligand so that steric hindrance may be avoided. This linker arm may be attached to the liposome or the hormone, preferably to the liposome. In this case, the use of a single coupling agent is preferred for linking the linker arm, as fixed to the liposome or to the hormone, to the

Before linking a coupling agent to the hormone it may be highly desirable to protect the receptor-binding site of the hormone if one or several amino acid residues which may be involved in the linkage should be located in this region.

This may be conventionally achieved by random partial protection of functional groups of amino acid residues of the hormone. Such a method is for example, reported in Jansons et al, Analytical Biochemistry 111: 54 (1981) as it discloses a process for linking antibodies to liposomes which comprises random partial protection of NH_2 functional groups of lysine residues of the antibody with citraconic anhydride before linking the antibody to liposomes. Once the linkage is achieved, the blocking agent is then removed. However, due to the random nature of the protection process, the percentage of antibody-liposome conjugates obtained by this method which have conserved a fully effective antigen binding site is rather low.

It has now been found that very effective protection of the binding site of the hormone is achieved by using a monoclonal antibody (MAb) specific for the binding site. Accordingly, the invention further provides a process for coupling liposomes to a hormone which comprises reversibly protecting the binding site of the hormone with an antibody specific for the binding site. After linkage of the hormone to the liposomes, or in any event after performing any reaction step that might otherwise result in interference with or blocking of the binding site, the antibody is removed from the binding site to yield a site capable of interacting with the appropriate cell receptors for the hormone.

More particularly, the binding site of IL-2 which is located between amino acid residues 20-30 of the molecule may be protected by a MAb which specifically recognizes this area. Such a MAb is commercially available from Genzyme Inc. under the reference DMS-1. Thus, for use in the present invention, the MAb is coupled to a stationary phase e.g., a resin or a gel. Then IL-2 is applied to the

Figure 8 shows a reaction for coupling phosphatidylethanolamine to camphoquinone-10-sulphonic acid.

Example 1: Protection and modification of IL-2

A protein-A agarose resin is reacted with a MAb specific for the receptor binding site of IL-2 (available from Genzyme Inc; catalog reference DMS-1) by washing the resin with PBS buffer, applying the MAb to the resin, crosslinking the MAb to the resin in the presence of 12.5% glutaraldehyde in PBS for 60 min at 4°C and finally washing the resin again with buffer.

Then 50 µg of IL-2 in 100 µl buffer is applied to the resin coupled to the MAb, and modified with SMPB as indicated in Figure 3. SMPB is added to IL-2 in the molar ratio of 25:1. SMPB specifically reacts with the ϵNH_2 groups of lysine residues. The modified IL-2 is then eluted from the resin with 2M NaSCN in 0.05M Tris, pH 8 followed by a second elution treatment with 6% betaine in 0.2N acetic acid, all at 4°C.

An IL-2 receptor binding assay shows that the resulting SMPB is able to bind to the IL-2 receptor of target cells. Furthermore, in an ELISA assay, SMPB modified IL-2 is recognized by the monoclonal antibody to the receptor binding domain of IL-2, as is unmodified IL-2 eluted from the column under the same conditions.

In an alternative procedure, MBS (maleimidobenzoyl-N-hydroxy succinimide ester, a derivative of SMPB) is also used to modify IL-2. Use of this reagent also yields biologically active IL-2 when assayed in a receptor binding assay and in the anti-IL-2 ELISA.

Example 2: Synthesis of SATA-DSPE (See Fig. 4)

125 mg of fresh succinimidyl-S-acetylthioacetate (SATA) is mixed with 75 mg of distearoyl phosphatidylethanolamine (DSPE) in a round bottom flask. Then 15 ml CHCl_3 :MeOH (1:1) is added, followed by 100-135 µl triethylamine. The flask is then flushed with nitrogen and

10 mg of mixture of DSPC, cholesterol and SATA-DSPE in the above ratio is dissolved in 1 ml CHCl_3 , dried to a film under nitrogen and then further dried under vacuum overnight.

The lipid film is dispersed in 1 ml PBS buffer pH 8.3, left at 65°C for 60 min, sonicated with a probe sonicator for 6 min at 65°C and held at the same temperature to form SUVs. The SUVs are then centrifuged at 13,000 xg for 10 min to remove any particles of titanium from the sonicator probe.

0,9 ml of the SUV preparation (about 10 mg of lipids) is then applied to a Sephacryl S300 column to remove the free SATA-DSPE (not incorporated into liposomes). The column is first presaturated with DSPC:cholesterol (2:1) SUVs in PBS, 1 mM EDTA 0,2 mM PMSF to maximise the recovery of SATA-DSPE SUVs in the column flow through. SATA-DSPE SUVs are recovered in a volume of 2.4 ml containing approximately 3,3 mg/ml of lipids. SUVs produced in this manner have a diameter of about 50 nm.

Example 3 b): Preparation of drug-containing liposomes

Example 3 a) is repeated except that the PBS buffer used to disperse the lipid film contains 55 mg/ml of methotrexate. SATA-DSPE SUV's containing methotrexate are obtained.

Example 4: Attachment of IL-2 to liposomes

The modified SMPB-IL-2 of Example 1 is covalently coupled through its maleimido groups to the SUVs of Example 3 a) or 3 b).. With reference to Figure 5, the coupling is achieved as follows:

The SUVs containing SATA-DSPE are first activated by deacetylating the SATA residue so that it can react with SMPB-IL-2. 10 μl of freshly prepared 0,5M hydroxylamine is added to 1 ml of the SUV preparation of Example 1, (10 mg/ml in PBS). Incubation is performed for 30 min at 22°C under argon.

After purification, the activated lipid is incorporated into liposomes whereby drug or marker is entrapped in standard fashion. The liposomes are then ready for reaction with free protein sulfhydryls either present on the unmodified protein, or with derivatives prepared as described below.

For example, SATA may be used in limiting concentration. Thus, to a solution of IL-2 at about pH 8 may be added a solution of SATA in DMF or dioxane. The amount of substitution may be controlled by limiting the quantity of reagent. The sulfhydryl is then deblocked by the addition of hydroxylamine and the IL-2 immediately coupled to liposomes having the maleimido group on the surface.

Example 6: Modification of IL-2 at selected (limited) lysine residues

The most abundant and available protein reaction sites are lysine ϵNH_2 groups. Since some of the lysine residues are located at the receptor binding site, any attempt to use the lysine residues for coupling must comprise limiting the lysine residues which actually undergo a reaction.

If the reactivity of the critical lysine residues is much faster than or at least comparable to most of the other lysine residues, the method shown in Figure 7 may be employed. In this method essentially all but a few lysine residues are first derivatized with a reversible blocking agent. Using only enough reagent so that the most reactive or accessible lysines are blocked, or after blocking followed by partial deblocking, the remaining lysine residues are then derivatized by using a large excess of a coupling agent, e.g. SATA. To a solution of IL-2 in borate buffer at pH 8.8 is added citraconic anhydride, maintaining the pH by addition of 1 N sodium hydroxide. The citraconic acid groups can be removed at pH 2, and this reaction can be interrupted by again raising the pH. SATA is added in excess to the solution at pH 8.8, derivatizing all free amines. The excess reagent is removed by dialysis and the remaining citraconic acid

used; for total in vivo uptake by target cells nitrilotriacetic acid (NTA) is preferably used.

In a conventional biodistribution study, SUVs of a given composition are prepared in the presence of chelating agent and Indium-111 for passive encapsulation of indium, or the ionophore A23187 for subsequent active encapsulation of Indium-111 into preformed liposomes. In either situation the liposomes are separated from unencapsulated materials by treatment with additional EDTA, followed by column chromatography.

Biodistribution measurements are conducted by injecting the study animals intravenously with the ^{111}In -liposome/IL-2 complex. At several times after injection (for example at 1, 3, 6 and 24 hours), animals are sacrificed and tissues collected for gamma counting. Liposomes loaded with ^{111}In -EDTA may be used for initial biodistribution studies. This tightly bound chelate complex allows measurement of in vivo retention times for the liposomes. Experiments performed relating to the present invention have shown that ^{111}In -EDTA alone is rapidly cleared in vivo. Therefore, any recovered Indium-111 activity may be interpreted as ^{111}In -liposome/IL-2.

Uptake of liposome/IL-2 by lymphocytes in a test animal may be studied using the ^{111}In -NTA complex. This relatively weak chelate complex has been shown to maintain Indium-111 in the aqueous compartment of liposomes, but quickly to release Indium-111 once the chelate is in the proximity of protein or carbohydrate macromolecules. Therefore, treatment of study animals with ^{111}In -NTA-liposome/IL-2 formulations provides a means for measuring cumulative uptake by lymphocytes.

This may be accomplished by treating animals with the ^{111}In -NTA formulations. Test animals may be given single or multiple (2-3) injections of the ^{111}In -NTA liposomes by either intravenous or intraperitoneal routes of administration. At various times after treatment, animals are sacrificed and lymphocytes isolated from, for

animal models in which T-lymphocytes can be reproducibly activated. Two models that are readily available are the rat skin allograft model and the rat burn model (30% body surface). Using either model, groups of animals would receive either liposome/IL-2-containing the immunosuppressive drug, or only liposome/IL-2. Retention of the allograft as the animal undergoes a protocol of liposome/IL-2 treatment is then measured. Those receiving liposome/IL-2 without the liposome-incorporated drug will readily reject the graft due to presence and cytolytic activity of activated T-cells in the area of the graft. Alternatively, liposome/IL-2 containing a drug of choice may also be used in the course of either the rat or mouse leukemia model. Tests have shown that the liposome/IL-2 complex of Example 4 is attracted to activated T cells in vitro.

The present delivery vehicles may be used in a variety of therapeutic contexts, including mammalian disease therapy and in vivo diagnosis, and biodistribution studies have shown significant advantages. When practiced using small unilamellar liposomes, which have been shown both to be useful in targeting solid tumors and to have greater circulation times than other vehicles, the present delivery vehicles may be used to deliver medicinal agents or diagnostic markers (as for example radioactive labels, fluorescent molecules and NMR-imaging agents such as magnetite) to neoplastic cells or particular organs of the body such as the liver. As contemplated herein, where the cell population of interest is characterized by a cellular receptor such as the interleukin-2 receptor, the present delivery vehicles may be utilized to deliver cytotoxic, regulatory, diagnostic or other molecules in a targeted manner. Thus, in the case of the liposome/IL-2 delivery vehicle of the present invention, active agents may be delivered to lymphocytes so as to treat, regulate or diagnose conditions involving malfunction of the immune system (including genetic or non-genetic autoimmune diseases such as rheumatoid arthritis, juvenile onset diabetes, systemic lupus erythematosus and others, as well as transplantation responses such as graft-versus-host disease), lymphocyte-related cancers (including lymphomas and leukemias such as adult or chronic

Claims: -

1. Liposomes which are coupled to a hormone having a biologically active cell receptor binding site.
2. Liposomes according to claim 1 in which the hormone is a cytokine.
3. Liposomes according to claim 2 in which the cytokine is interleukin-2 (IL-2).
4. Liposomes according to any one of claims 1 to 3 which encapsulate at least one cytotoxic drug.
5. Liposomes according to any one of claims 1 to 4 the membrane of which comprises phosphatidylethanolamine.
6. Liposomes according to any one of claims 1 to 5 which are coupled to the hormone by a coupling moiety comprising a SATA and a SMPB residue said SATA residue being linked to the liposomes and said SMPB residue being linked to the hormone.
7. Liposomes according to any one of claims 1 to 5 which are coupled to the hormone by a coupling moiety comprising a 4 to 6 carbon linker arm.
8. Liposomes according to any one of claims 1 to 7 which are small unilamellar vesicles.
9. Liposomes according to any one of claim 1 to 8 which are coupled to the hormone at an amino acid residue on the hormone which is not located at the receptor binding site and which is selected from lysine, cysteine, arginine and histidine residues.
10. A process for coupling liposomes to a hormone having a biologically active receptor binding site which comprises either

- a) fixing a coupling agent to liposomes and then reacting with the hormone, or
- b) fixing a coupling agent to the hormone and reacting with liposomes or,
- c) fixing a first coupling agent to liposomes, fixing a second coupling agent to the hormone and then reacting the coupling moieties together.

11. A process according to claim 10 which comprises fixing to the liposomes a coupling agent which is selected from succinimidyl-4-(p-maleimidophenyl)-butyrate (SMPB), and sulphur-containing succinimidyl compounds.

12. A process according to claim 11 which comprises fixing succinimidyl-S-acetylthioacetate (SATA) to liposomes.

13. A process according to claim 10 which comprises fixing to the hormone a coupling agent which is selected from succinimidyl-4-(p-maleimidophenyl)butyrate (SMPB), sulpho-SMPB, N-(4-carboxy-cyclohexyl-methyl) maleimide (SMCC), sulpho-SMCC, M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), sulpho-MBS, N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB) and sulpho-SIAB.

14. A process according to claim 13 which comprises fixing succinimidyl-4-(p-maleimidophenyl)-butyrate (SMPB) to the hormone.

15. A process according to claim 10 which comprises fixing SATA to liposomes, fixing SMPB to the hormone and then reacting the coupling moieties together.

16. A process according to any one of claims 10 to 15 in which the cell receptor binding site of the hormone is reversibly protected before fixing the coupling agent.

AMENDED CLAIMS

[received by the International Bureau on 2 November 1989 (02.11.89)
original claim 1 amended ; other claims unchanged (1 page)]

1. Liposomes which are covalently coupled to a hormone having a biologically active cell receptor binding site.
2. Liposomes according to claim 1 in which the hormone is a cytokine.
3. Liposomes according to claim 2 in which the cytokine is interleukin-2 (IL-2).
4. Liposomes according to any one of claims 1 to 3 which encapsulate at least one cytotoxic drug.
5. Liposomes according to any one of claims 1 to 4 the membrane of which comprises phosphatidylethanolamine.
6. Liposomes according to any one of claims 1 to 5 which are coupled to the hormone by a coupling moiety comprising a SATA and a SMPB residue said SATA residue being linked to the liposomes and said SMPB residue being linked to the hormone.
7. Liposomes according to any one of claims 1 to 5 which are coupled to the hormone by a coupling moiety comprising a 4 to 6 carbon linker arm.
8. Liposomes according to any one of claims 1 to 7 which are small unilamellar vesicles.
9. Liposomes according to any one of claim 1 to 8 which are coupled to the hormone at an amino acid residue on the hormone which is not located at the receptor binding site and which is selected from lysine, cysteine, arginine and histidine residues.
10. A process for coupling liposomes to a hormone having a biologically active receptor binding site which comprises either

FIGURE 2

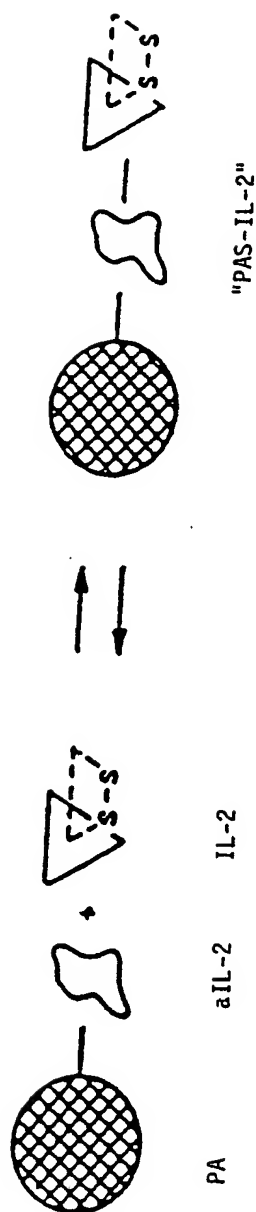


FIGURE 4

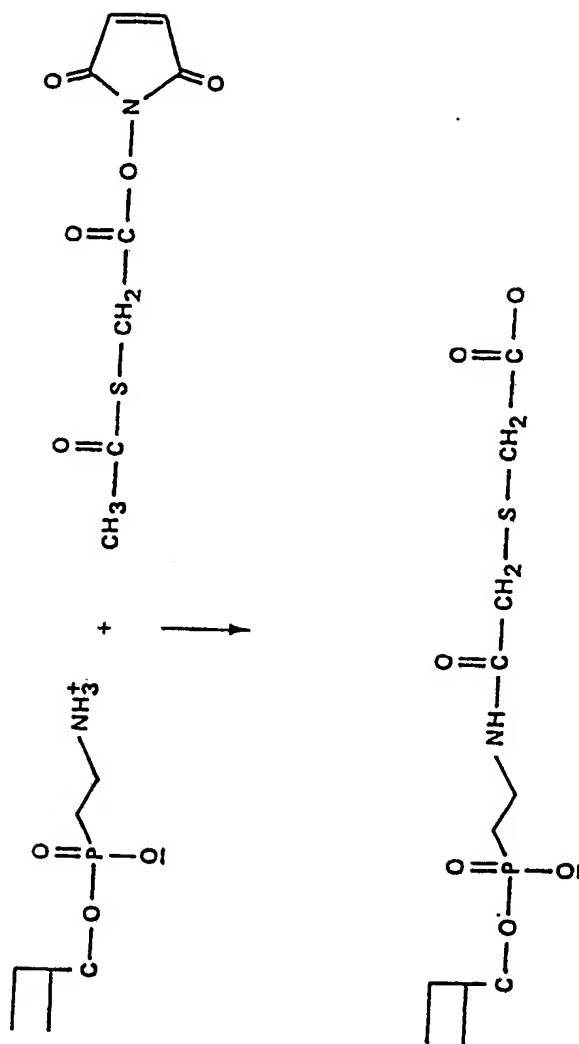
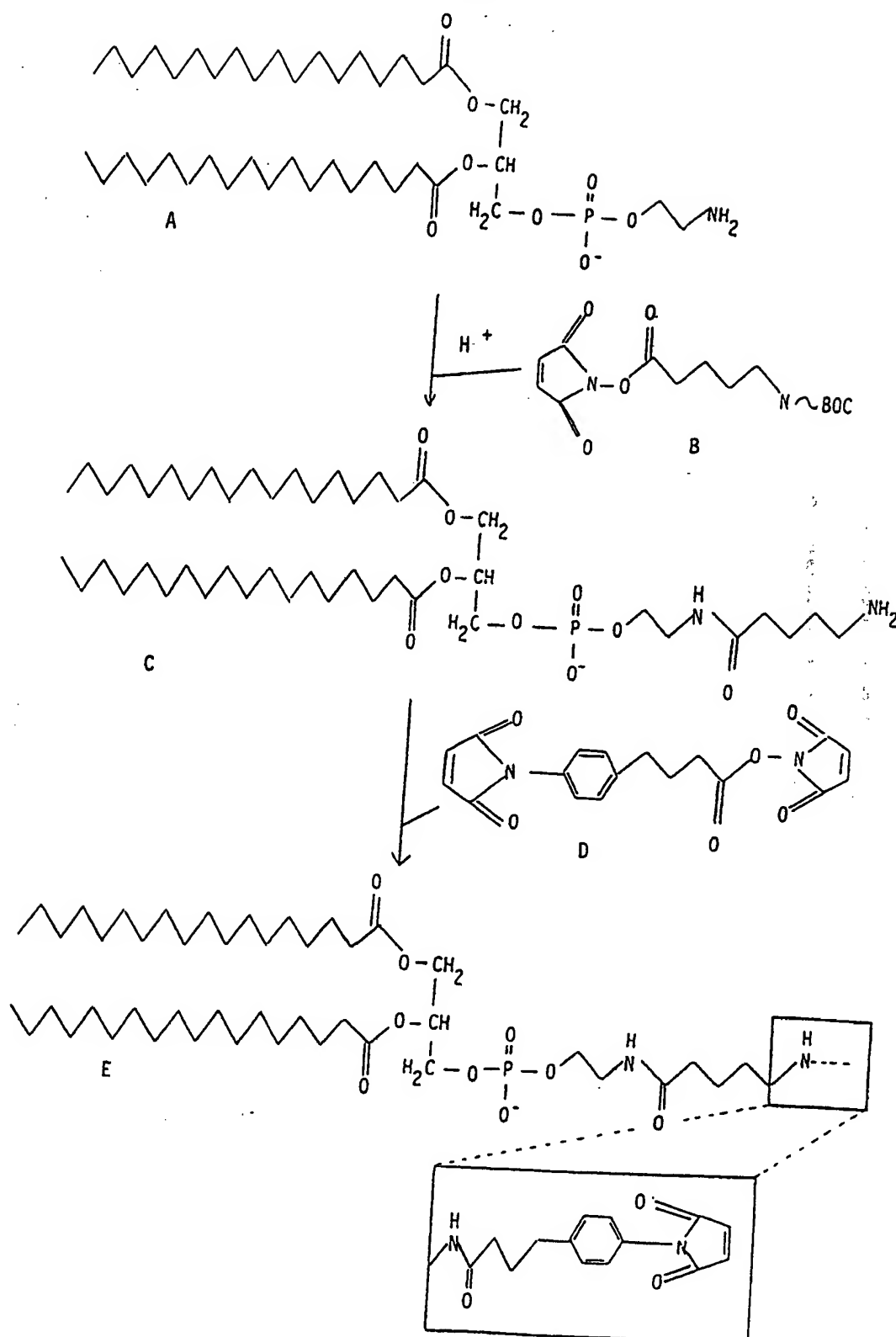
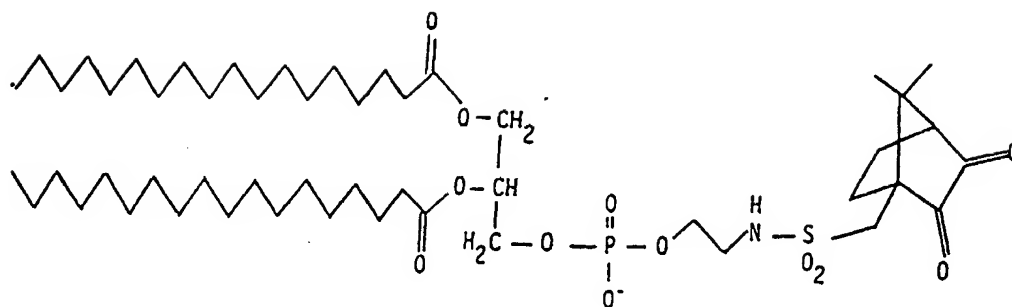
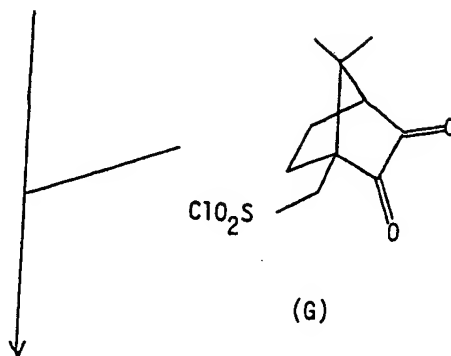
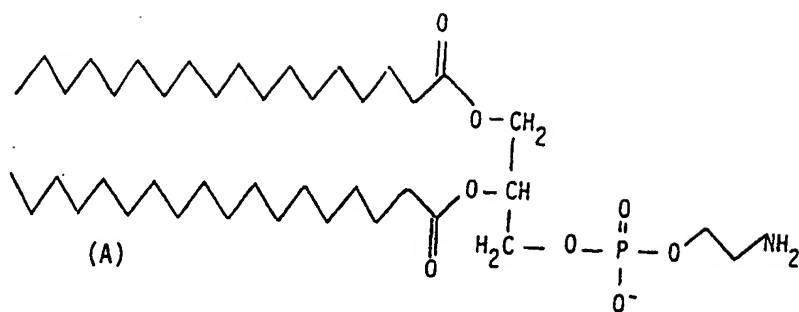


Figure 6



SUBSTITUTE SHEET

Figure 8



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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 8900521
SA 28546

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